

**Amendments to the Specification:**

Please replace paragraph 10 with the following amended paragraph:

The methods provided include mutagenesis of iron hydrogenase proteins including mutagenesis of the  $X^1X^2X^3FX^4X^5X^6GGVMEAAX^7R$  (SEQ ID NO:185) and  $ADX^8TIX^9EE$  (SEQ ID NO:186) segments. In some methods, cognate sequences of these conserved segments of iron hydrogenases are substituted into a Chlamydomonas iron hydrogenase. In some methods, gene reassembly methods are performed in which a Chlamydomonas iron hydrogenase is mutagenized by incorporation of segments of iron hydrogenase proteins from other species. Preferred segments for inclusion in gene reassembly include segments that form parts of the gas channel, also referred to as the gas channel. In some methods a higher molecular weight amino acid is substituted into a gas channel segment, such as a tryptophan for the methionine in the C. reinhardtii TIMEE (SEQ ID NO:142) segment. In other gene reassembly methods the iron hydrogenase is reassembled using methods that involve attaching sections of duplex DNA that have only one overhanging nucleotide. In other methods oligonucleotides encoding gas channel segments are annealed to a scaffold nucleic acid, where the oligonucleotides anneal to non-overlapping sites. Preferably, the mutagenesis of a hydrogenase does not decrease the protein's ability to accept electrons from an electron donor. In some methods the mutagenized nucleic acid is transcribed by a light-driven promoter.

Please replace paragraph 29 with the following amended paragraph:

Figure 15 shows the key to the identity of the amino acids of step 1 of figure 13 and the corresponding identity of codons in nucleic acids in steps 2-9 of figures 13-14. Row 1 of Figure 15 uses SEQ ID NO:185 as an example. Row 2 uses SEQ ID NO:187 as an example.

Please replace paragraph 30 with the following amended paragraph:

Figure 16 shows the divergent sequences from SEQ ID Nos: 1-112 that correspond to the segments of Iron ~~hydrogenases hydrogenases~~ hydrogenases that line the gas channel. These are the segments that are schematically depicted in figure 13, step 1. The sequences are used to design

the oligonucleotides in step 2 of figure 13. Column 1 shows SEQ ID NOs: 124-141 listed as segments 1 through 18, respectively. Column 2 shows SEQ ID NOs: 142-147 listed as segments 1 through 6, respectively.

Please replace paragraph 31 with the following amended paragraph:

Figure 17 shows one example of how gas channel segments from SEQ ID Nos: 1-112 are reverse translated into recoded nucleotide sequence. *C. reinhardtii* flanking sequence is added to each side of the oligonucleotide sequence to ensure adequate annealing. Although step 1 of figure 13 depicts 3 segments, which figure 16 shows only 2 segments, the  $X^1X^2X^3FX^4X^5X^6GGVMEAAX^7R$  (SEQ ID NO:185) segment is broken into two distinct segments to allow greater combinatorial diversity ~~af~~ of the library, as this figure shows. Exemplary amino acid segments depicted include DLTIWEEGT (SEQ ID NO:189), TIWEE (SEQ ID NO:144, which is a subsequence of SEQ ID NO:2), DLTIMEEGT (SEQ ID NO:190, which is a subsequence of SEQ ID NO:26), GAGAIFGATGGV (SEQ ID NO:191, which is a subsequence of SEQ ID NO:23), MEAASRT (SEQ ID NO:192, which is a subsequence of SEQ ID NO:98), LGSGAGVLFGTGGV (SEQ ID NO:193, which is a subsequence of SEQ ID NO:26), MEAALRTAYE (SEQ ID NO:194, which is a subsequence of SEQ ID NO:26), LGSGAGAIFGATGGV (SEQ ID NO:195, which is a chimera of SEQ ID NOs:191 and 193), MEAALRSAYE (SEQ ID NO:196, which is a chimera of SEQ ID NOs:192 and 196), gacctgacctctgggaggaggcacc (SEQ ID NO:197, which encodes the amino acid segment of SEQ ID NO:189), ctgggcagcggcgccggcgccatcttcggcgccaccggcggtg (SEQ ID NO:198, which encodes the amino acid segment of SEQ ID NO:195), and atggaggccgcctgcgcagcgctacgag (SEQ ID NO:199, which encodes the amino acid segment of SEQ ID NO:196).

Please replace paragraph 91 with the following amended paragraph:

In one embodiment, the *C. reinhardtii* iron hydrogenase gene is mutated to alter amino acid residues in and near the gas channel. The gas channel is a section of iron hydrogenases, depicted in figure 9, that allows newly formed hydrogen molecules to leave the protein. Oxygen irreversibly inactivates the active site of iron hydrogenases by entering the active site through the

gas channel (for background see Ghirardi, Appl Biochem Biotechnol (1997) 63-65: 141-151). Because hydrogen molecules are smaller than oxygen molecules, narrowing the gas channel using methods ~~deisclosed~~ disclosed herein provides iron hydrogenases that are not inactivated by oxygen. Preferably, substitutions of residues that are in and near the gas channel generate side chains that are of higher molecular weight or are longer than the side chain at that position in the wild type protein. Such substitutions are preferable because they narrow the gas channel and block the entry of oxygen into the active site. As one nonlimiting example, residues in the highly conserved X<sup>1</sup>X<sup>2</sup>X<sup>3</sup>FX<sup>4</sup>X<sup>5</sup>X<sup>6</sup>GGVMEAAX<sup>7</sup>R (SEQ ID NO:185) segment can be mutated. This segment forms a turn followed by an alpha helix. The F corresponds to Phe234 in the wild type *C. reinhardtii* iron hydrogenase. The X residues are highly variable between iron hydrogenase from different species. For example, the X<sup>4</sup>X<sup>5</sup>X<sup>6</sup> residues are GVT, GAT, GVS, GNS, CAS, and numerous other sequences in different iron hydrogenases. Nonetheless, members of the iron hydrogenase family usually have a G as the first residue of this triplet. Although the GGVMEA (SEQ ID NO:187) amino acid motif is highly conserved among members of the iron hydrogenase family, there are some iron hydrogenases that have variant sequences corresponding to this motif. For example, the *D. fructosovorans* iron hydrogenase (GenBank Accession number D57150) has the sequence GGVI EAA (SEQ ID NO:188). Thus, even highly conserved motifs that surround the gas channel are tolerant of change.

Please replace paragraph 92 with the following amended paragraph:

Other amino acid motifs also form secondary structures near the gas channel. For example, the ADX<sup>8</sup>TIX<sup>9</sup>EE (SEQ ID NO:186) motif is in close contact with the channel. In particular, the T, I and X<sup>9</sup> residues are near the channel.

Please replace paragraph 93 with the following amended paragraph:

In one embodiment, highly variable amino acids are subjected to saturation mutagenesis. In another embodiment, highly variable amino acids are substituted with any amino acid that is of a higher molecular weight than the wild type amino acid at that position in either of the *C. reinhardtii* iron hydrogenases. In another embodiment, variable amino acids in either of the *C.*

reinhardtii iron hydrogenases are substituted with amino acids that are found in the corresponding position in iron hydrogenases from different species. In yet another embodiment, the  $X^1X^2X^3FX^4X^5X^6GGVMEAAX^7R$  (SEQ ID NO:185) motif is mutated in either of the C. reinhardtii iron hydrogenases referred to as hydA and hydB (Forestier, Eur J Biochem. 2003 Jul;270(13):2750-8), wherein some of the X residues are substituted with amino acids that are found in the corresponding position in iron hydrogenases from different species while other X residues are substituted with residues that are not found in any known species. In one embodiment residues  $X^1X^2X^3$  are from species 1, residues  $X^4X^5X^6$  are from species 2, and residue  $X^7$  is from species 3, where these X residues are placed in the context of a C. reinhardtii iron hydrogenase protein, and where none of species 1, 2, or 3 is C. reinhardtii. The methods provided herein include mutagenizing genes by substituting any segment of a protein sequence into another protein sequence, including genes encoding iron and nickel-iron hydrogenase proteins. Preferable lengths for segments include 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more amino acids. Of course, the methods provided also included substituting single amino acids from one species into the proteins of another species at a particular position as well as substituting amino acids that do not correspond to amino acids of another species at a particular position.

Please replace paragraph 124 with the following amended paragraph:

In another embodiment, promoter sequences from a plurality of genes in the genome of an organism are used to transform cells, followed by screening or selection for a desired phenotype. For example, a plurality of 500, 1000, 1500, 2000, or more base pair promoters are amplified from the C. reinhardtii genome. The full genome sequence has been completed and can be found in databases maintained by the Joint Genome Institute, Walnut Creek, CA at <http://genome.jgi-psf.org/chlr1/chlr1-home.html>. The promoter sequences are connected to a selectable marker sequence and used to transform the nuclear and/or chloroplast and/or mitochondrial genome. The surviving transformants are screened or selected for a desired phenotype. Preferably, the transformants are screened for a phenotype related to a metabolic function such as the ability to produce hydrogen. Optionally, independent transformants of

promoter ~~constructs~~ constructs that produce an increased amount of hydrogen are mated and the progeny are screened for a further increased amount of hydrogen over any of the parents. The mating can be ~~pairwise~~ pairwise or multiparental.